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(54) **Methods of assay.**

(57) An electrochemical specific-binding assay of a ligand wherein one of the components is redox-modified by having incorporated into its molecular structure a redox centre, and which includes the step of determining the extent to which an electrochemical characteristic of the components is perturbed by complex formation and/or by controlled external influences.

Redox centres are molecules, atoms, groups, ions, radicals etc capable of undergoing reduction and oxidation. A preferred example is ferrocene or its derivatives.

The electrochemical apparatus will typically contain a working electrode, which is preferably solid. Components may advantageously be immobilised onto such a solid working electrode.

When controlled external influences are employed, these may comprise displacement of non-immobilised electroactive components relative to the working electrode.

The method is particularly applicable to the assay of antigens, haptens and antibodies.

The use of redox centres avoids radioactive labels and provides a simple, rapid, sensitive and specific assay method.

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Methods of Assay

The present invention relates to methods of assay of one of a pair of specific binding partners, and to kits of reagents for carrying out these methods.

5       There is today a great need for rapid and accurate methods of assaying biologically active substances (which may be at low concentration), particularly in body fluids such as blood, saliva or urine. A wide variety of medical conditions,  
10 such as pregnancy, drug overdose, metabolic birth defects, hormonal disorders and diabetes can be diagnosed using such assay techniques.

Many assay methods rely on the formation of a complex between the species under assay (hereinafter  
15 called "ligand") and another species to which it will bind specifically (hereinafter called "specific binding partner"). The extent of complex formation is a function of the amount of the ligand present.

The assay of ligand is determined by monitoring  
20 the extent of complex formation, for example by the use of chemical or biochemical labels. Several methods of labelling have been employed, for example radioisotopic or enzyme labelling, spin-labelling or labelling employing fluorescent or bioluminescent  
25 species. Although each of these known methods may be advantageous in certain respects, it has been found in practise that, in general, only radioisotopic labelling gives a method which is sufficiently rapid, sensitive and specific, and thus it is that  
30 technique which has become most widely used.

There are, however, serious disadvantages to the use of radioactive labels. Radioactive labels have limited shelf life due to spontaneous decay, necessitating frequent recalibration of

the equipment, and their use will require adherence to strict safety precautions and is subject to legal regulation.

Furthermore, a separation step may be necessary following the formation of the labelled complex in order to remove unbound labelled component from the system before determination of the amount of labelled complex by the usual counting methods.

We have now found that, by employing a redox centre as herein defined as a label and measuring a perturbation in an electrochemical characteristic of the components, a simple, rapid, sensitive and specific method of quantitative or qualitative ligand assay may be obtained, which avoids the use of radioactive species.

Thus, in its broadest aspect, the invention provides a method of assay of a ligand in a sample using an electrochemical apparatus containing components comprising

- (a) the sample,
  - (b) a specific binding partner to the ligand, and
  - (c) if desired, at least one reagent selected from ligand analogues and specific binding partners,
- at least one of the said components other than the sample being redox-modified (as herein defined);

which method includes the step of determining whether, and, if desired, the extent to which an electrochemical characteristic of the components is perturbed by complex formation and/or by controlled external influences.

The assay can be completed from the determined perturbation with reference to calibration data.

The term "redox-modified" used herein refers to the modification of a molecular structure by incorporation of at least one redox centre. The redox centre is an organic, inorganic or organometallic

species capable of undergoing, under suitable conditions, reduction and oxidation. Redox centres may, for example, be complete molecules, portions of molecules, atoms, ions, radicals, complexes or polymers.

- 5 Typically, the redox-centre will undergo both oxidation and reduction during the period of the assay, thereby returning at the end of the assay to its initial oxidation level.

The term "ligand analogue" used herein refers  
10 to a species capable of complexing with the same specific binding partner as the ligand under assay, and includes inter alia within its scope a known quantity of the ligand species under assay.

The electrochemical apparatus contains a  
15 working electrode, an auxiliary electrode and optionally a reference electrode. The working electrode will preferably be solid and have a working surface of e.g. carbon (preferably graphite e.g. pyrolytic graphite), or metal e.g. silver, gold or platinum.  
20 A suitable electrochemical apparatus is illustrated in Figure 3a of the accompanying drawings. The working electrode 1 is composed of an elongate core 2 (e.g. of brass) tipped with a working surface 3 (e.g. of pyrolytic graphite) and having a coating  
25 4 (e.g. of epoxy resin). The auxiliary (counter) electrode 5 is suitably of platinum. A calomel reference electrode 6 shown, connected to the cell via a luggin capillary 7.

Although the order of introduction of the  
30 components into the apparatus is not critical, it is preferable that a complex is formed after introduction of the final component but not prior thereto. It is, however, also possible for there to be complex present before the final component  
35 is added, in which case the final component will become complexed by displacing one component of the complex.

A variety of electrochemical methods exploiting

any two of the three parameters potential (E),  
current (i) and time (t) may be used to measure  
electrochemical characteristics of the components.

For example, electrochemical measurements can be  
5 made using differential pulse voltammetry, cyclic  
voltammetry or square-wave voltammetry. When cyclic  
voltammetry is used, a circuit such as, for example,  
that shown in Figure 3b of the accompanying drawings  
may be employed. In this Figure, C represents  
10 the auxiliary (counter) electrode, W the working  
electrode and R the reference electrode. This  
circuit may conveniently be used in conjunction  
with apparatus of the type shown in Figure 3a,  
the electrochemical current i being measured.

15 In a typical case, formation of the complex  
perturbs the electrochemical behaviour of the redox  
centre, the degree of perturbation being a function  
of the proximity of the site of complex formation  
to the redox centre. It is not necessary for a  
20 full voltammogram to be determined in the measurements  
made; it may be sufficient, for example, for an  
appropriate poised potential to be selected and  
readings of current taken at that point. The degree  
or rate of perturbation can then be related to  
25 the amount of ligand present in the sample, from  
calibration data obtained with similar systems  
using known amounts of ligand.

It may be found, on measuring the perturbation  
of an electrochemical characteristic, that no,  
30 or only a slight, perturbation is observed due  
to complex formation. In that case, it may prove  
advantageous artificially to generate or enhance  
a perturbation by controlled external influences.  
Although the magnitude of the external influence  
35 may have some bearing on the change induced, and  
must therefore be consistent with any such influence  
employed in calibration experiments, it is thought  
that any change produced in the perturbation remains

a function of the ligand/binding partner complex.

The artificial generation or enhancement of the perturbation is preferably performed by displacement of the complex relative to the working electrode, for example by further complexing the complex with a species which will bind specifically to the complex, coupled to a solid support, or by using a specific binding partner coupled to a solid support, with subsequent displacement of the support and coupled molecules. In extreme cases, the displacement may constitute complete removal of the complex from the apparatus, but in general the complex will be displaced within the apparatus. Other methods for artificially generating or enhancing the perturbation include, for example, the use of antibodies to the redox-centre (e.g. anti-ferrocene antibodies) to quench the electrochemical characteristic of the redox-centre, or removing excess uncomplexed redox-modified reagent, e.g. by draining from the apparatus or by coupling to a suitable solid support and removing the said solid support from the apparatus.

In a method of the invention exploiting the time (t) parameter, the rate of perturbation of the electrochemical characteristic as a result of complex formation may be determined. Conveniently, the initial rate of perturbation will be measured. Such a method is applicable to a competitive assay in which the ligand and a redox-modified ligand analogue compete for complexing with the specific binding partner. The reaction may be viewed as competitive inhibition of the ligand complexing reaction by the ligand analogue. Thus, the initial rate of perturbation is related to the concentration of ligand present and from a calibration plot of the initial rate of perturbation v. concentration of ligand present, the ligand assay can be readily determined.

The method of assay involving a determination of the rate of perturbation is also applicable to non-competitive assays where the redox-modified ligand analogue is absent and sufficient redox-modified specific binding partner is employed to enable all the ligand introduced to be complexed. Artificial generation or enhancement of the perturbation as herein described may if desired be carried out before the rate of perturbation is determined.

10 In another competitive assay, not involving a rate determination, an initial reading of the appropriate electrochemical characteristic will be made before the addition of a final component which may initiate the complexing reaction or may  
15 displace a component from a complex already present. On completion of complex formation, a final reading of the electrochemical characteristic of the components will be taken and the perturbation determined. By reference to calibration data of perturbations  
20 with known ligand concentrations the amount of ligand in the sample can be determined.

The method of the invention also enables a non-competitive assay, not involving a rate determination, to be performed.

25 So long as there is sufficient specific binding partner present, the absolute perturbation, or the rate of perturbation, in the electrochemical characteristic of the components from the initial reading before introduction of the final component  
30 will be dependent on the amount of added ligand and the assay can therefore be calculated with reference to calibration data.

The methods of the present invention are generally simpler than known methods, in that they  
35 may eliminate the need for the separation of complexed and uncomplexed phases before the assaying step.

If electrode-immobilised components are employed, the technique is further simplified in that the

need for separate addition of the component to the electrochemical apparatus is eliminated. Additionally, the direct interaction between the electrode and the electrode-immobilised species may lead to an improvement in the sensitivity of the perturbation measurements.

According to one embodiment of the present invention, therefore, there are provided methods of assay of a ligand in a sample as hereinbefore defined wherein the working electrode of the electrochemical apparatus is solid and one of the components other than the sample is immobilised thereon.

The immobilised component may thus be a redox-modified or unmodified ligand analogue or specific binding partner.

It will be appreciated that, in this embodiment, due to the resulting complex being coupled to the electrode, it is not possible artificially to generate or enhance a perturbation by displacement of the resulting complex relative to the working electrode. However, a perturbation may still be artificially generated or enhanced, for example by complexing any uncomplexed redox-modified reagent remaining in solution with a species which will complex specifically with the reagent, coupled to a solid support, with subsequent displacement of the support and coupled molecules.

The invention will be particularly described hereinafter with reference to an antibody or an antigen as the ligand. However the invention is not to be taken as being limited to assays of antibodies or antigens. Examples of ligands which may be assayed by the method of the invention are given in Table I below, together with an indication of a suitable specific binding partner in each instance.

Table I

|    | Ligand                         | Specific Binding Partner     |
|----|--------------------------------|------------------------------|
| 5  | antigen                        | specific antibody            |
|    | antibody                       | antigen                      |
|    | hormone                        | hormone receptor             |
|    | hormone receptor               | hormone                      |
|    | polynucleotide                 | complementary polynucleotide |
| 10 | strand                         | strand                       |
|    | avidin                         | biotin                       |
|    | biotin                         | avidin                       |
|    | protein A                      | immunoglobulin               |
|    | immunoglobulin                 | protein A                    |
| 15 | enzyme                         | enzyme cofactor (substrate)  |
|    | enzyme cofactor<br>(substrate) | enzyme                       |
|    | lectins                        | specific carbohydrate        |
|    | specific carbohydrate          | lectins                      |
| 20 | of lectins                     |                              |

The method of the invention has very broad applicability but in particular may be used to

25 assay: hormones, including peptide hormones (e.g. thyroid stimulating hormone (TSH), luteinising hormone (LH), follicle stimulating hormone (FSH), insulin and prolactin) or non-peptide hormones (e.g. steroid hormones such as cortisol, estradiol,

30 progesterone and testosterone, or thyroid hormones such as thyroxine (T4) and triiodothyronine), proteins (e.g. human chorionic gonadotrophin (hCG), carcino-embryonic antigen (CEA) and alphafetoprotein (AFP)), drugs (e.g. digoxin), sugars, toxins or vitamins.

It will be understood that the term "antibody" used herein includes within its scope

- a) any of the various classes or sub-classes of immunoglobulin, e.g. IgG, IgM, derived from any of the animals conventionally used, e.g. sheep, rabbits, goats or mice,
- b) monoclonal antibodies,
- c) intact molecules or "fragments" of antibodies, monoclonal or polyclonal, the fragments being those which contain the binding region of the antibody, i.e. fragments devoid of the Fc portion (e.g., Fab, Fab', F(ab')<sub>2</sub>) or the so-called "half-molecule" fragments obtained by reductive cleavage of the disulphide bonds connecting the heavy chain components in the intact antibody.

The method of preparation of fragments of antibodies is well known in the art and will not be described herein.

- The term "antigen" as used herein will be understood to include both permanently antigenic species (for example, proteins, bacteria, bacteria fragments, cells, cell fragments and viruses) and haptens which may be rendered antigenic under suitable conditions.

Incorporation of the redox centre into the molecular structure of an antibody may be achieved, for example, by any of the following methods:

- (i) providing the redox centre with one or more functional groups capable of bonding interactions with the molecular structure of the antibody;
- (ii) using cross-linking groups;
- (iii) using the avidin-biotin binding system, (e.g. avidin-labelled antibody binding with biotin-labelled ferrocene molecules or biotin-labelled antibody binding with avidin-labelled ferrocene);
- (iv) using an antibody conjugated with a reagent (e.g. fluorescein isothiocyanate (FITC))

binding with a second antibody raised to the reagent (e.g. anti-FITC antibody) to which is coupled ferrocene;

- (v) using a second antibody labelled with ferrocene,  
5 said second antibody being raised to the first antibody.

Similar methods may be applied as desired for the incorporation of a redox centre into an antigen molecule. Suitable methods are known in  
10 the art and will not be discussed in detail here. For example, the incorporation of ferrocene into certain steroids was described in Journal of Organo-metallic Chemistry, 160 (1978) pp. 223-230.

Immobilisation of an antibody or antigen  
15 molecule onto the electrode may be effected by various methods. The attachment of the antibody or antigen to the electrode can be via any portion of the molecular structure so long as specific immunological activity is retained at the antibody  
20 or antigen binding site.

Thus, for example, electrode-immobilisation of unmodified antibody or antigen reagent may be achieved by bonding interactions between functional groups on the antibody or antigen molecule and  
25 the electrode, or by cross-linking or adsorption onto the surface of the electrode. Binding of reagents to the electrode may be accomplished by methods analogous to known methods for binding such reagents to solid supports (e.g. particles  
30 and beads), for example those described in published European Patent Application No. 105714.

Electrode-immobilisation of redox-modified reagent may, for example, be achieved by any of the following methods:

- 35 (i) incorporating a redox centre into the molecular structure of free reagent and subsequently immobilising the reagent onto an electrode at a site remote from the redox centre in

the same way as described above for unmodified reagents;

- (ii) incorporating a redox centre into the molecular structure of a pre-immobilised reagent;
- 5 (iii) incorporating a bifunctional redox centre into the molecular structure of free antibody or antigen so as to enable one function to interact with the electrode: or
- 10 (iv) incorporating a bifunctional redox centre onto the electrode, so as to enable one function to interact with the molecular structure of free antibody or antigen.

The preferred redox centre for modifying antibodies or antigens is the organometallic 'sandwich' compound ferrocene (bis- $\eta^5$ -cyclopentadienyl iron (II)) or a derivative thereof. Ferrocene is desirable for this purpose because it is relatively cheap, non-toxic and provides an easily reversible system which in its reduced  $\text{Fe}^{\text{II}}$  state is not susceptible to oxidation by oxygen in the atmosphere. Examples of suitable ferrocene derivatives include functionalised derivatives, polymeric forms ('polyferrocenes') such as  $(\text{ferrocene})_4$  and 'boron tetraferrocene' ( $\text{B}(\text{ferrocene})_4$ ).

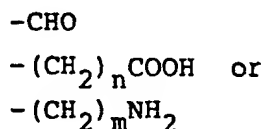
25 Examples of other redox centres which may be employed include nitroxides, viologens, derivatives of phenazine methosulphate or phenazine ethosulphate, cytochrome c, flavins, quinones, metal carbonyls (such as, for example, chromium carbonyl or molybdenum carbonyl) and their derivatives, and transition metal Schiff's base derivatives.

35 As indicated above, the redox centre may require functionalisation in order to permit successful binding to the antibody or antigen and/or to the electrode or to improve the electrochemical or other properties of the redox centre. For example, the redox potential of ferrocene is +442 mV vs NHE. By introducing functional groups onto the

ring system, this figure can be varied between +300 and +650 mV. Moreover, the water-solubility of carboxyl-substituted ferrocene is greater than that of the parent compound (see, for example,

- 5 R. Szentrinay, 1977 Amer. Chem. Soc. Symposium Series, 38, 154).

Thus, for example, in the case of ferrocene, it may be necessary to modify the ferrocene complex by providing one or both of the cyclopentadienyl  
10 groups with one or more side chains, e.g. of the formula



- 15 where n and m may be e.g. from 0 to 6. Additional functional groups may be incorporated into the side chain, typically those groups used in the chemical modification of proteins, for example mercuric chloride, precursors of nitrenes and carbenes,  
20 diazo or iodide groups. The terminal -CHO, -COOH or -NH<sub>2</sub> groups are then available to interact with suitable sites in the antibody or antigen molecule or on the electrode. The length of the side chain (i.e. the value of n or m) will depend on the structure  
25 of the antibody or antigen to which the ferrocene is to be bound. Similar functionalisation may be desirable when redox centres other than ferrocene are used.

The electrochemical apparatus preferably  
30 contains an aqueous assay medium comprising inter alia, pH buffer. Means may be provided for incubating the assay mixture at any desired temperature.

In a further feature of the present invention, therefore, there are provided kits of reagents  
35 and apparatus for carrying out the assays of the invention. Suitable kits may comprise an electrochemical apparatus containing at least one electrode; and

an aqueous assay medium with suitable redox-modified or unmodified reagent present (either in solution or immobilised on the working electrode). Other components (e.g. further reagents etc) and the  
5 sample to be assayed may conveniently be introduced through an entry port provided in the apparatus.

Advantageously the apparatus may be pre-calibrated and provided with a scale whereby the perturbation in electrochemical characteristic of the components  
10 may be read off directly as an amount of antibody or antigen in the sample.

In kits employing the competitive assay method, the competing redox-modified ligand analogue reagent may preferably be initially mixed with the sample  
15 to be assayed outside the apparatus and the mixture then introduced into the apparatus.

As illustration of various aspects of this invention, antibody and antigen assays which may be performed according to the invention are given  
20 in Table II below. These assays are given purely for illustration and are in no way limiting of the present invention. It will be appreciated that the assays give in Table II may, where appropriate, be carried out either by an absolute or by a rate  
25 method as described above.

Ab = antibody    \*= redox-modified species  
Ag = antigen    \_ = species under assay (in sample)  
(lim) = known, limited amount of reagent

Table II

A. Assay of Antigen

|    |  |  |
|----|--|--|
| 5  | Components of apparatus immediately after initial measurement of electro-chemical characteristic | Subsequent assay steps   |
| 10 | 1.<br><br>[ELECTRODE] Ab*  | a) <u>Ag</u> added to form Ab*: <u>Ag</u> complex<br>b) optional generation or enhancement of perturbation<br>c) measurement of electro-chemical characteristic<br>d) calculation of assay from perturbation |
| 15 |  |  |
| 20 | 2.<br><br>[ELECTRODE] <u>Ag</u>  | a) Ab* added to form Ab*: <u>Ag</u> complex<br>b) optional generation or enhancement of perturbation<br>c) measurement of electro-chemical characteristic<br>d) calculation of assay from perturbation       |
| 25 |  |  |
| 30 | 3.<br><br>[ELECTRODE]-Ab*  | a) <u>Ag</u> added to form immobilised Ab*: <u>Ag</u> complex<br>b) measurement of electro-chemical characteristic<br>c) calculation of assay from perturbation  |

|    |  |  |
|----|--|--|
| 4. |  | a) <u>Ag</u> (having two roomly-spaced determinants to which the antibody reagents are separately directed) added to form [ELECTRODE]-Ab: <u>Ag</u> :Ab* complex |
| 5  | [ELECTRODE]-Ab Ab*   |  |
|    |  | b) optional generation or enhancement of perturbation  |
| 10 |  | c) measurement of electrochemical characteristic   |
|    |  | d) calculation of "two-site" assay from perturbation   |
| 15 | Note: This assay method may be carried out using the "forward", "simultaneous" or reverse" techniques. |  |
| 5. |  | a) Ab (lim) added to give a competitive system   |
| 20 | [ELECTRODE]-Ag*(lim)   | b) measurement of electrochemical characteristic   |
|    | <u>Ag</u>  | c) calculation of assay from perturbation.   |
| 25 |  |  |
| 6. | [ELECTRODE]-Ab(lim)  | a) Ag*(lim) and <u>Ag</u> added together to give competitive system  |
| 30 |  | b) optional generation or enhancement of perturbation  |
|    |  | c) measurement of electrochemical characteristic   |
| 35 |  | d) calculation of assay from perturbation  |

|    |    |                          |   |
|----|----|--------------------------|---|
| 5  | 7. | [ELECTRODE]    Ab(lim)   | a) <u>Aq</u> and Ag* added to<br>give a competitive system<br>b) optional generation<br>or enhancement of perturbation<br>c) measurement of electro-<br>chemical characteristic<br>d) calculation of assay<br>from perturbation |
| 10 |    |                          |   |
| 15 | 8. | [ELECTRODE]-Ag <u>Aq</u> | a) Ab* (lim) added to give<br>a competitive svstem<br>b) generation or enhancement<br>of perturbation<br>c) measurement of electro-<br>chemical characteristic<br>d) calculation of assay<br>from perturbation                  |

B. Assay of Antibody

|    |   |
|----|---|
| 5  | <p>Components of apparatus<br/>immediately after initial measurement of characteristic</p> <p>Subsequent assay steps</p>  |
| 10 | <p>9. [ELECTRODE] Ag*</p> <p>a) <u>Ab</u> added to form <u>Ab:Ag*</u> complex<br/>b) optional generation or enhancement of perturbation<br/>c) measurement of electrochemical characteristic<br/>d) calculation of assay from perturbation</p>  |
| 20 | <p>10. [ELECTRODE] <u>Ab</u></p> <p>a) Ag* added to form <u>Ab:Ag*</u> complex<br/>b) optional generation or enhancement of perturbation<br/>c) measurement of electrochemical characteristic<br/>d) calculation of assay from perturbation</p> |
| 30 | <p>11. [ELECTRODE]-Ag*</p> <p>a) <u>Ab</u> added to form immobilised <u>Ab:Ag*</u> complex<br/>b) measurement of electrochemical characteristic<br/>c) calculation of assay from perturbation</p>   |

|    |   |  |
|----|---|--|
| 5  | 12.<br>[ELECTRODE]-Ab <u>Ab</u>   | a) Ag* (having two roomly-spaced determinants to which the antibody reagents are separately directed) added to form [ELECTRODE]-Ab:Ag*: <u>Ab</u> complex. |
| 10 |   | b) measurement of electro-chemical characteristic<br>c) calculation of "two-site" assay from perturbation  |
| 15 | Note: This assay method may be carried out using the "forward", "simultaneous" or "reverse" techniques. |  |
| 20 | 13.<br>[ELECTRODE] Ab*(lim)<br><u>Ab</u>  | a) Ag(lim) added to give competitive system<br>b) measurement of electro-chemical characteristic<br>c) calculation of assay from perturbation.             |
| 25 | 14.<br>[ELECTRODE]-Ab*(lim)<br><u>Ab</u>  | a) Ag(lim) added to give competitive system<br>b) measurement of electro-chemical characteristic<br>c) calculation of assay from perturbation              |

|    |     |                          |  |
|----|-----|--------------------------|--|
| 5  | 15. | [ELECTRODE]-Ag(lim)      | a) $Ab^*(lim)$ and <u>Ab</u> added together to give competitive system |
| 10 |     |                          | b) optional generation or enhancement of perturbation                  |
|    |     |                          | c) measurement of electro-chemical characteristic                      |
|    |     |                          | d) calculation of assay from perturbation                              |
| 15 | 16. | [ELECTRODE] Ag(lim)      | a) <u>Ab</u> and $Ab^*(lim)$ added to give a competitive system        |
| 20 |     |                          | b) optional generation or enhancement of perturbation                  |
|    |     |                          | c) measurement of electro-chemical characteristic                      |
|    |     |                          | d) calculation of assay from perturbation                              |
| 25 | 17. | [ELECTRODE]-Ab <u>Ab</u> | a) $Ag^*(lim)$ added to give a competitive system                      |
| 30 |     |                          | b) optional generation or enhancement of perturbation                  |
|    |     |                          | c) measurement of electro-chemical characteristic                      |
|    |     |                          | d) calculation of assay from preturbation                              |

The following non-limiting Examples are intended to illustrate the invention more fully

EXAMPLE 1

5 Assay of Thyroxine (T4) using a redox-modified antigen with enhancement of perturbation using controlled external influences

In this assay free redox-modified antigen is measured after competition between modified  
10 and unmodified antigen for a fixed number of antibody binding sites.

Preparation of Starting Materials

(i) Ferrocene-modified thyroxine (T4) hormone

15 To stirred, cooled (-10°C) dimethylformamide (3 mls), was added ferrocene monocarboxylic acid (FMCA) (1 m mole) and then triethylamine (1.0 m mole) and isobutylchloroformate (1.0 m mole). The mixture was then further cooled to -15°C and stirred for  
20 30 minutes and then allowed to warm up to room temperature and stirred for a further hour. The resulting carboxycarbonic anhydride of ferrocene was added dropwise to a solution of T4 in sodium hydroxide/ethanol and stirred for 24 hours, after  
25 which the sample was filtered then concentrated by rotary evaporation. The resulting precipitate was washed in carbon tetrachloride then ethyl acetate several times, the insoluble ferrocene monocarboxylic acid-T4 conjugate (T4-FMCA) then being filtered  
30 out of the ethyl acetate solution.

(ii) Purification of Ferrocene-modified thyroxine conjugate (T4-FMCA)

The T4-FMCA conjugate was purified by high

performance liquid chromatography using C18 reverse phase column. After purification the T4 and ferrocene concentrations in the conjugate were measured by radioimmunoassay and electrochemistry respectively.

5 (iii) Characteristics of the T4-FMCA conjugate

Incubation of T4-FMCA with excess anti-T4 antibody showed that 73.4% of the T4-FMCA was bound to the antibody.

10 The electrochemistry of the conjugate was also compared with that of the T4 and FMCA. The conjugate shows two peaks on the forward wave of the cyclic voltammogram at a pyrolytic graphite electrode (Voltage Scan Rate =  $20\text{mVs}^{-1}$ ), the first being at +310 mV vs a standard calomel electrode  
15 (S.C.E.) (the ferrocene peak), the second at +410 mV vs S.C.E. (T4) (Figure 1). On the return wave only one peak at a potential of +255 mV vs S.C.E. was observed. Cyclic voltammograms at pyrolytic graphite electrodes (Voltage Scan Rates =  $5\text{mVs}^{-1}$ )  
20 of the unmodified components of the conjugate show that FMCA (0.2 mM FMCA in Tris (50mM; pH 7.4)) has peaks at +320 mV and +260 mV vs S.C.E. for the forward and return waves respectively (Figure 2a) whilst T4 exhibits a single peak at +420 mV  
25 vs S.C.E. on the forward wave (Figure 2b).

(iv) Solid Phase reagent comprising anti-T4 polyclonal antibodies covalently linked to magnetisable cellulose particles.

30 Anti-T4 was a conventional polyclonal antiserum obtained by immunising sheep with T4 conjugated to a high molecular weight protein. The magnetisable cellulose particles were a composite of cellulose containing approximately 50% black ferric(ous) oxide ( $\text{Fe}_3\text{O}_4$ ), with mean particle diameter of 3

micron (see Forrest and Rattle, "Magnetic Particle Radioimmunoassay", in Immunoassays for Clinical Chemistry, p. 147-162, Ed. Hunter and Corrie, Churchill Livingstone, Edinburgh (1983)). Anti-T4 antiserum  
5 was covalently coupled to the magnetisable cellulose following cyanogen bromide activation of the cellulose, according to the procedure of Axen et al, Nature 214, 1302-1304 (1967). The antiserum was coupled at a ratio of 1 ml antiserum to 1 gram of magnetisable  
10 solid phase. Anti-T4 magnetisable solid phase was diluted to a concentration of 25 g/ml in Tris-HCl buffer, 50 mM, pH 7.4.

(v) Apparatus used to measure the electrochemistry of T4-FMCA.

15 Cyclic voltammetry was performed in a three electrode electrochemical cell using a pyrolytic graphite working electrode (Figures 3a and 3b).

(vi) Standard solutions of T4

T4 (sodium salt) was obtained from Sigma  
20 London Chemical Company, England. Standard solutions were made by dissolving the T4 in sodium hydroxide (0.1 M) then diluting with Tris-HCl buffer (50 mM pH 7.4) to the desired concentration.

(vii) Assay procedure for T4

25 Duplicate samples were run, in which 50  $\mu$ l of T4 standard was added to 100  $\mu$ l of T4-FMCA ( $1.3 \times 10^{-5}$  M) and mixed, after which 100  $\mu$ l anti-T4 antibody coupled to solid phase was added. The mixture was incubated at room temperature for  
30 30 minutes with constant agitation and then the solid phase particles were separated magnetically. The resulting supernatant was transferred to the

electrochemical cell where the current at a potential of +310 mV vs standard calomel electrode (S.C.E.) was measured. An example current/T4 concentration curve is shown in Figure 4. Electrochemical current  $i$  in amps  $\times 10^{-8}$  is plotted along the vertical axis whilst T4 concentration (in  $\mu$  moles per litre) is plotted along the horizontal axis.

EXAMPLE 2

10

Assay of human chorionic gonadotrophin (hCG) using a redox-modified antibody with enhancement of perturbation using controlled external influences

15 Preparation of starting materials

(i) Redox-modified anti-hCG monoclonal antibodies

Monoclonal antibodies were obtained from mouse ascites fluid by the process reported by Milstein and Kohler in Nature 256 495-497 (1975).  
20 Antibodies from individual hybridoma cell lines were screened to identify those producing antibody to discrete antigenic determinants. Those having the highest affinities to hCG were selected for use in the assay.

25 Ferrocene carboxaldehyde (FCA) was used to introduce a redox centre into the structure of an anti-hCG monoclonal antibody, antibody A. The incorporation of the FCA into the antibody was achieved by formation of a Schiff's base between  
30 the active aldehyde group of the ferrocene and a free amino group on the antibody.

Ferrocene carboxaldehyde (0.15 mg per ml in sodium carbonate buffer 1 M per litre, pH 9.5) was incubated with 0.27 mg per ml of antibody A  
35 (also in sodium carbonate buffer) for 2 hours at 22°C in the dark with constant mixing. The resulting

conjugate was purified by gel filtration. The fractions were examined electrochemically and only those fractions whose cyclic voltammogram at a pyrolytic graphite electrode (with a Voltage Scan of  $10\text{mVs}^{-1}$ ) exhibited the forward and return waves characteristic of the ferrocene redox reaction (as shown in Figure 5a of the accompanying drawings) were used in the assay, those antibody fractions having no incorporated ferrocene (see Figure 5b) being discarded.

(ii) Preparation of anti-hCG (antibody B) conjugated to fluorescein isothiocyanate (FITC)

A second monoclonal antibody to hCG (antibody B) specific for a different antigenic determinant was conjugated to FITC.

Conjugation of FITC to monoclonal antibody was achieved by reacting 200  $\mu\text{g}$  fluorescein isothiocyanate (FITC), Sigma London Chemical Co., England, with 5 mg antibody in 1.4 ml sodium bicarbonate buffer, 0.2 M, pH 9.0, for 18 hours at room temperature. The reaction mixture was purified by gel filtration on Sephadex G-50 superfine, giving a product incorporating an average of 6 molecules FITC per antibody molecule.

(iii) Preparation of anti-FITC antibody covalently coupled to magnetisable solid phase

Anti-FITC was a conventional polyclonal antiserum obtained by immunising sheep with FITC conjugated to keyhole limpet haemocyanin. The magnetisable cellulose particles were a composite of cellulose containing approximately 50% black ferric(ous) oxide ( $\text{Fe}_3\text{O}_4$ ), with mean particle diameter of 3 micron (see Forrest and Rattle, "Magnetic Particle Radioimmunoassay", in Immunoassays for Clinical

Chemistry, p. 147-162, Ed. Hunter and Corrie, Churchill Livingstone, Edinburgh (1983)). Anti-FITC antiserum was covalently coupled to the magnetisable cellulose following cyanogen bromide activation of the cellulose, according to the procedure of Axden et al, Nature 214, 1302-1304 (1967). The antiserum was coupled at a ratio of 2 ml antiserum to 1 gram of magnetisable solid phase.

Anti-FITC magnetisable solid phase was diluted to 100 mg per ml in Tris-HCl buffer (10 mM per litre, pH 7.4).

(iv) Preparation of hCG standard solutions

A freeze dried preparation of hCG, calibrated against the first international reference preparation (75/537) was obtained from Biodata S.p.A., Milan, Italy. This sample was diluted in buffer Tris-HCl, (10 mM, pH 7.4) to the desired concentration.

(v) Apparatus used for electrochemical measurement

The electrochemical apparatus was the same equipment as that of Example 1.

(vi) Assay procedure for hCG

An immunometric immunoassay using the redox-modified antibody A was used to measure hCG.

Fixed volumes (100  $\mu$ l) of hCG standard were mixed with 100  $\mu$ l of antibody A (10  $\mu$ g per ml protein) and 100  $\mu$ l of antibody B (6  $\mu$ g per ml protein). After a 30 minute incubation period at room temperature the electrochemical current was measured ( $t=0$ ) after which 20  $\mu$ l of anti-FITC antibody was added and incubated for a further five minutes with constant agitation. The application of an external magnetic field removed the solid phase and attached immunocomplex

from solution, the electrochemical current due to any ferrocene remaining in the supernatant being measured (t=5).

The electrochemical signal was defined as:

$$\begin{array}{l} 5 \\ \text{signal unit} = \frac{(i_{t=0}) - (i_{t=5}) \text{ for sample}}{(i_{t=0}) - (i_{t=5}) \text{ for zero standard}} \end{array}$$

where i = electrochemical current measured at +330 mV vs S.C.E.. The zero standard was an analagous  
10 system containing no hCG. A plot of signal versus hCG concentration (in International Units per ml) is shown in Figure 6 of the accompanying drawings.

CLAIMS

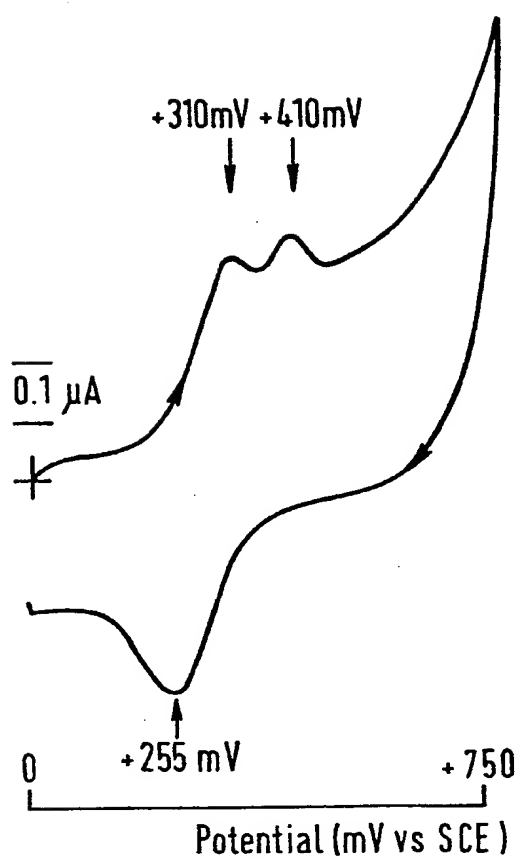
1. A method of assay of a ligand in a sample using an electrochemical apparatus containing components comprising
  - (a) the sample
  - 5 (b) a specific binding partner to the ligand, and
  - (c) if desired, at least one reagent selected from ligand analogues and specific binding partners,
- 10 at least one of the said components other than the sample being redox-modified (as herein defined); which method includes the step of determining whether, and, if desired, the extent to which an electrochemical characteristic of the components
- 15 is perturbed by complex formation and/or controlled external influences.
2. A method as claimed in claim 1, wherein the electrochemical apparatus contains a solid working electrode.
- 20 3. A method as claimed in claim 1 or claim 2, wherein the controlled external influence, when present, comprises displacement of the complex formed relative to the working electrode of the electrochemical apparatus, or removal of the complex
- 25 from the apparatus.
4. A method as claimed in claim 2, wherein one of the components other than the sample is immobilised on the working electrode.
5. A method as claimed in any one of claims
- 30 1, 2 and 4, wherein the controlled external influence, when present, comprises displacement of excess uncomplexed redox-modified component relative

to the working electrode of the electrochemical apparatus, or removal of excess uncomplexed redox-modified component from the apparatus.

6. A method as claimed in any preceding claim,  
5 wherein the or each redox-modified component contains at least one redox centre formed from ferrocene or a functionalised derivative thereof.
7. A method as claimed in claim 6, wherein the functionalised derivative of ferrocene contains  
10 one or more side chains of the formula  $-\text{CHO}$ ,  $-(\text{CH}_2)_n\text{COOH}$  or  $-(\text{CH}_2)_m\text{NH}_2$  (where  $n$  and  $m$  are each from 0 to 6).
8. A method as claimed in any preceding claim,  
15 wherein the electrochemical characteristic is a peak current observed under the application of a preselected potential across the components.
9. A method as claimed in any preceding claim wherein the ligand is an antigen or an antibody.
10. A kit of reagents and/or apparatus for carrying  
20 out a method of assay as claimed in any preceding claim.
11. A kit of reagents and/or apparatus as claimed in claim 10, which comprises an electrochemical apparatus containing at least one electrode and/or  
25 an aqueous assay medium with redox-modified and/or unmodified reagent, the said reagent being in solution or immobilised on the working electrode.

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FIG. 1.



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FIG. 2a.

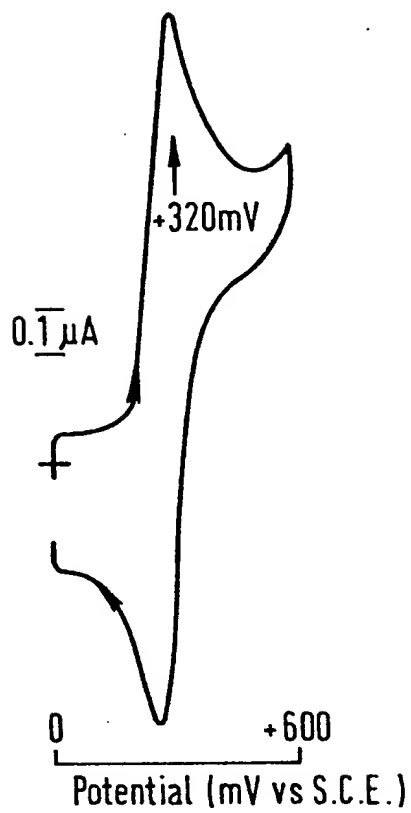
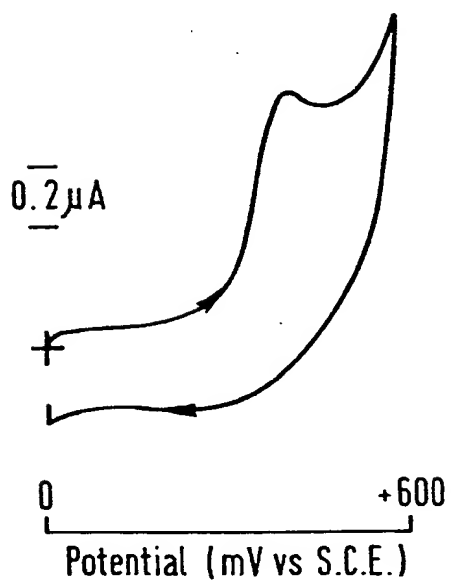


FIG. 2b.



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FIG. 3a.

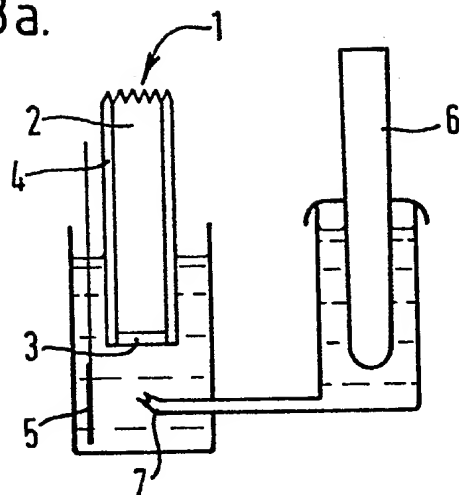
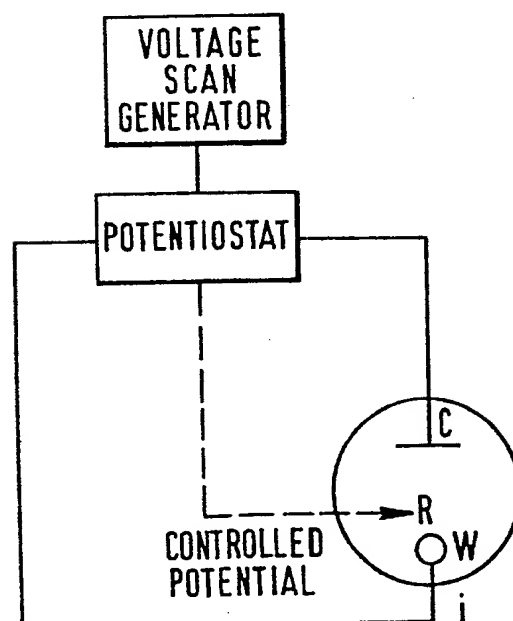


FIG. 3b.



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FIG. 4.

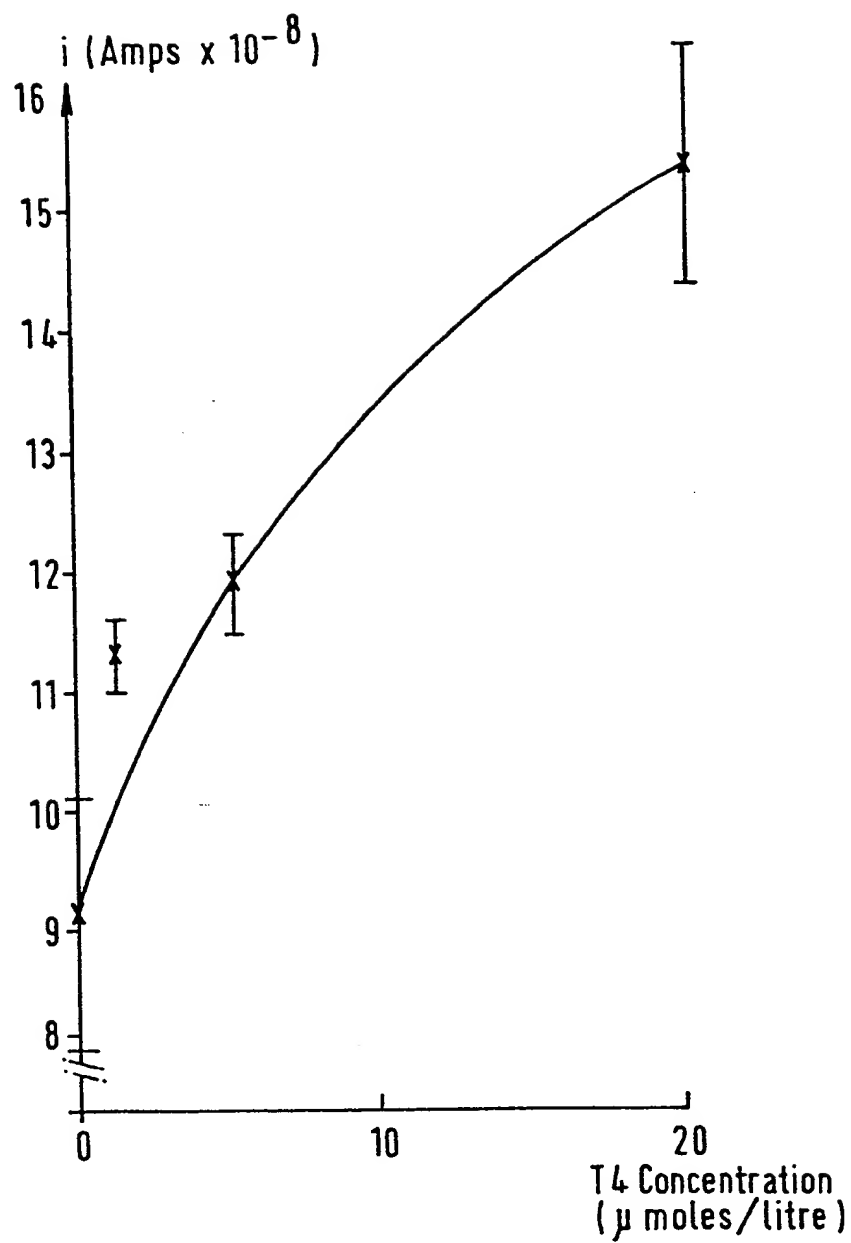


FIG. 5a.

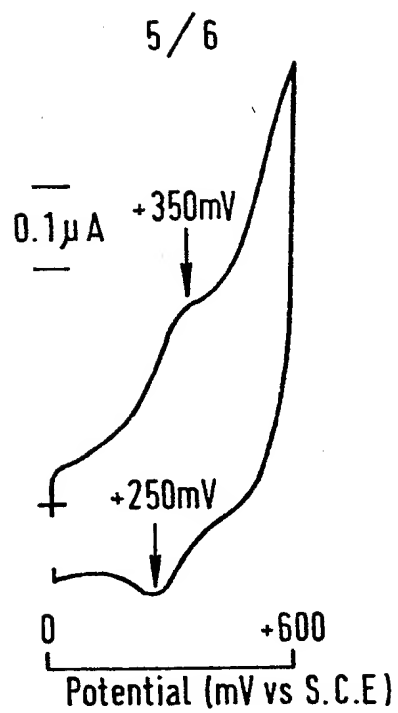
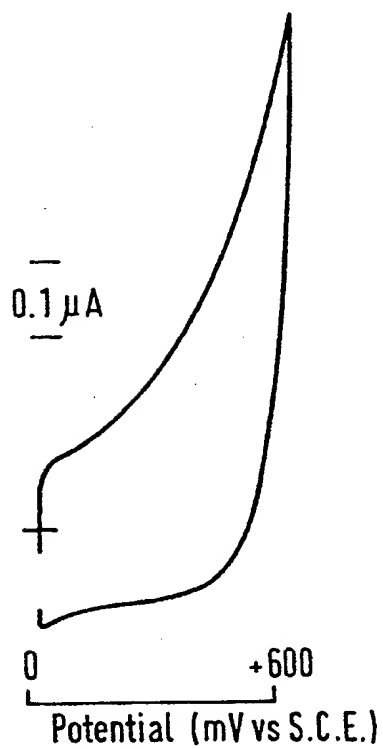
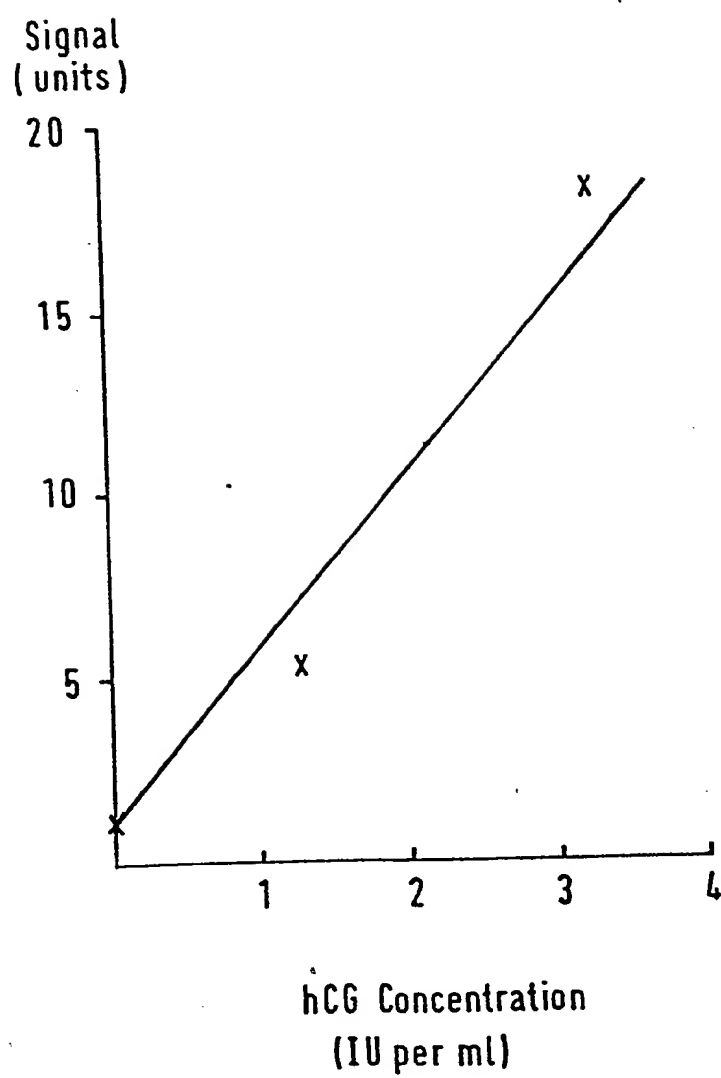


FIG. 5b.



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FIG. 6.



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